

## A NEW CONCEPT AFFECTING RESTORATION OF INFLAMMATION-REACTIVE ASTROCYTES

L. BLOCK,<sup>b</sup> U. BJÖRKLUND,<sup>a</sup> A. WESTERLUND,<sup>a</sup>  
P. JÖRNEBERG,<sup>c</sup> B. BIBER<sup>b</sup> AND E. HANSSON<sup>a\*</sup>

<sup>a</sup> Department of Clinical Neuroscience and Rehabilitation, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, SE 413 45 Gothenburg, Sweden

<sup>b</sup> Department of Anaesthesiology and Intensive Care Medicine, Institute of Clinical Sciences, The Sahlgrenska Academy, University of Gothenburg, SE 413 45 Gothenburg, Sweden

<sup>c</sup> Department of Anaesthesiology and Intensive Care Medicine, Division of Pain Management, NU-Hospitals, SE 461 85 Trollhättan, Sweden

**Abstract**—Long-lasting pain may partly be a consequence of ongoing neuroinflammation, in which astrocytes play a significant role. Following noxious stimuli, increased inflammatory receptor activity, influences in  $\text{Na}^+/\text{K}^+$ -ATPase activity and actin filament organization occur within the central nervous system. In astrocytes, the  $\text{Ca}^{2+}$  signaling system,  $\text{Na}^+$  transporters, cytoskeleton, and release of pro-inflammatory cytokines change during inflammation. The aim of this study was to restore these cell parameters in inflammation-reactive astrocytes. We found that the combination of (1) endomorphin-1, an opioid agonist that stimulates the  $\text{G}_{i/o}$  protein of the  $\mu$ -opioid receptor; (2) naloxone, an opioid antagonist that inhibits the  $\text{G}_s$  protein of the  $\mu$ -opioid receptor at ultralow concentrations; and (3) levetiracetam, an anti-epileptic agent that counteracts the release of IL-1 $\beta$ , managed to activate the  $\text{G}_{i/o}$  protein and  $\text{Na}^+/\text{K}^+$ -ATPase activity, inhibit the  $\text{G}_s$  protein, and decrease the release of IL-1 $\beta$ . The cell functions of astrocytes in an inflammatory state were virtually restored to their normal non-inflammatory state and it could be of clinical significance and may be useful for the treatment of long-term pain.

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**Key words:**  $\mu$ -opioid receptor,  $\mu$ -opioid agonist,  $\mu$ -opioid antagonist, astrocyte networks, IL- $\beta$  release, levetiracetam.

### INTRODUCTION

During injury, pain can be dissociated from its normal physiological role and can persist for a long period of time, even after the primary noxious stimulus has passed (McMahon and Malcangio, 2009; Milligan and Watkins, 2009; Gao and Ji, 2010). The mechanisms behind insufficiently healed neuroinflammation and how the neuronal and non-neuronal activities evoked by painful stimuli and inflammation are processed in the brain and throughout the central nervous system (CNS) are not well understood.

Under conditions that lead to neuroinflammation in the nervous system,  $\text{Ca}^{2+}$  signaling in the astrocyte network is overactivated, which triggers the activation of astrocytes and microglia (Strokin et al., 2011). Several receptors are influenced, and the expression of some receptors, such as Toll-like receptor 4 (TLR4) (Forshammar et al., 2011; Stevens et al., 2013), are increased, whereas the responses of other receptors, such as opioid receptors (El-Hage et al., 2006; Block et al., 2012), are changed. In addition, there is an increased release of glutamate into the neural synapse, where astrocytes are the predominant players in clearing the extracellular space. The astrocyte uptake of excessive extracellular glutamate by membrane-bound glutamate transporters, such as GLAST and GLT-1, play a critical role in preventing glutamate excitotoxicity (Zhao et al., 2012). In addition, metabotropic and ionotropic glutamate receptors will be excited. The astrocyte metabotropic receptor mGluR5 activates  $\text{G}_q$  protein-coupled receptors, resulting in increased intracellular  $\text{Ca}^{2+}$  release (Hansson, 1994; Bradley et al., 2009), whereas the *N*-methyl-D-aspartic acid (NMDA) receptor subunit NR2B is expressed in states of increased neuronal excitability (Krebs et al., 2003) and in inflammation-reactive astrocytes (Lundborg et al., 2011; Gérard and Hansson, 2012). These changes in the glial cells can lead to pathogenic chronic neuroinflammation. Subsequently the neurons change their excitability and signaling.

Endomorphin-1, a selective endogenous  $\mu$ -opioid receptor agonist, is capable of interacting with the pertussis toxin-sensitive  $\text{G}_i/\text{G}_o$  protein (Connor and Christie, 1999), and increases the activity of  $\text{Na}^+/\text{K}^+$ -ATPase *in vitro* (Horvath et al., 2003). Similar results have been observed with morphine, which indirectly

\*Corresponding author. Address: Department of Clinical Neuroscience and Rehabilitation, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Per Dubbsgatan 14, 1tr, SE 413 45 Gothenburg, Sweden. Tel: +46-31-786-3363; fax: +46-31-342-2467.

E-mail address: [elisabeth.hansson@neuro.gu.se](mailto:elisabeth.hansson@neuro.gu.se) (E. Hansson).

**Abbreviations:** ANOVA, analysis of variance; AUC, areas under the curve; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HHBSS, Hank's HEPES-buffered saline solution; LPS, lipopolysaccharide; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Sap, saponine; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline; TLR4, Toll-like receptor 4.

enhances  $\text{Na}^+/\text{K}^+$ -ATPase activity by activating  $\mu$ -opioid receptors and  $\text{G}_i/\text{G}_o$  protein (Masocha et al., 2002). Naloxone in ultralow picomolar concentrations has potent antagonistic actions on the excitatory  $\mu$ -opioid receptor  $\text{G}_s$  protein. Higher concentrations antagonize both excitatory  $\text{G}_s$  proteins, and inhibitory  $\text{G}_i/\text{G}_o$  proteins. As a consequence ultralow picomolar concentrations of naloxone enhance the analgesic effects of  $\mu$ -opioid receptor agonists (Crain and Shen, 1995; Wang et al., 2005; Tsai et al., 2009). Naloxone also modulates the  $\text{Na}^+/\text{K}^+$ -ATPase activity (Forshammar et al., 2011). Ultralow concentrations of naloxone stimulate the activity of  $\text{Na}^+/\text{K}^+$ -ATPase through activation of phospholipase C resulting in increases in intracellular  $\text{Ca}^{2+}$  release. Higher concentrations have antagonistic effects on the  $\text{Na}^+/\text{K}^+$ -ATPase activity (Zhang et al., 2008; Forshammar et al., 2011).

Levetiracetam, an effective anti-epileptic drug, has in inflammation-reactive astrocyte models been shown to restore functional gap junction coupling (Stienen et al., 2010) by increasing the expression of connexin 43, the predominant gap junction protein, and decrease the enhanced IL-1 $\beta$  level (Haghikia et al., 2008).

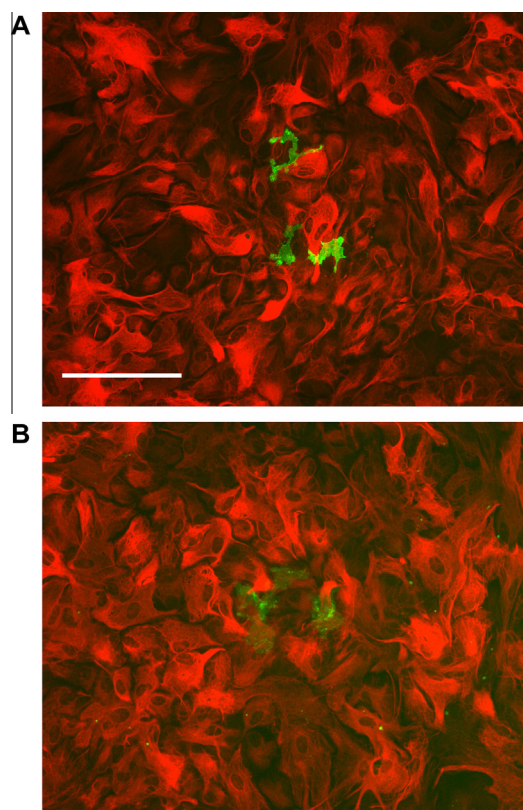
The aim of this study was to use endomorphin-1, naloxone, and levetiracetam to evaluate if they could counteract the increased astrocyte  $\text{Ca}^{2+}$  signaling, downregulation of the  $\text{Na}^+/\text{K}^+$ -ATPase activity, disorganization of the cytoskeleton, and increased release of IL-1 $\beta$ . These agents might have a potential to restore cell functions that are altered by inflammation in astrocytes back to a normal non-inflammatory level.

## EXPERIMENTAL PROCEDURES

In this study, the *in vitro* model involved astrocytes co-cultured with brain endothelial cells (Hansson et al., 2008). The biological rationale for co-culturing astrocytes with endothelial cells is that astrocytes are affected by substances that are released from the capillary endothelial cells of the BBB (blood–brain barrier) (Huber et al., 2001). These interactions are essential for a functional neurovascular system, where also the neurons take part (Abbott et al., 2006; Willis and Davis, 2008). The endothelial cells are not directly in physical contact with the astrocytes *in vitro*, and the interaction in the model is induced by the shared medium. The co-cultured astrocytes are morphologically differentiated by long, slender processes, and they exhibit greater  $\text{Ca}^{2+}$  responses and cytokine release than monocultured astrocytes. The  $\mu$ -opioid receptor is also better expressed in the co-cultured astrocytes (Hansson et al., 2008), as well as the TLR4 (Forshammar et al., 2011). Furthermore, the cultures have very few microglial cells, visualized with OX42 (Fig. 1A), as well as after treatment with lipopolysaccharide (LPS) for 24 h (Fig. 1B).

## Chemicals

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.



**Fig. 1.** Culture stained for OX42, a marker for microglial cells (green), and for GFAP, a marker for astrocytes (red). (A) Very few microglia were found in the co-cultured astrocytes. (B) Also very few microglia were found after treatment with LPS for 24 h. Scale bar = 50  $\mu\text{m}$ . Representative images are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The experimental protocols were approved by the Ethics Committee in Gothenburg for Laboratory Animals (Nos. 205-2010; 211-2010).

## Primary astrocyte cultures

The primary astrocyte cultures were prepared from newborn rat cerebral cortices (Charles River, Sulzfeldt, Germany) and cultured on glass coverslips (Nr 1, diameter 20 mm, BergmanLabora, Stockholm, Sweden) as described by Hansson et al. (2008).

## Microvascular endothelial primary cultures

Brain capillary fragments were isolated from male Sprague–Dawley rats, cerebral cortices (Charles River, 225–250 g), and the endothelial cells were cultured according to the protocol of Hansson et al. (2008).

## Astrocytes co-cultured with adult rat brain microvascular primary cultures

The experimental astrocytes were obtained after co-cultivation with primary brain microvascular endothelial cultures and primary astrocyte cultures. Astrocyte cultures at 6 d *in vitro* were co-cultured with newly prepared microvascular cultures. The endothelial cells

were grown on inserts above the astrocyte cultures. The cells from the two different cultures were never in contact. The cells were grown together for 9–11 d. At the time of the experiments, the astrocyte cultures were 15–17 d old, including 9–11 d of co-cultivation. The endothelial cells were removed before the experimental procedure (Hansson et al., 2008).

### Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (Bie & Berntsen, Herlev, Denmark) for 10 min and washed twice with phosphate-buffered saline (Invitrogen) containing 1% bovine serum albumin (PBS–BSA). The cells were permeabilized with PBS–BSA containing 0.05% saponine (PBS–BSA–Sap) for 20 min. Thereafter the cells were incubated for 1 h with a cocktail of rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark) and a mouse monoclonal antibody against OX42 (Serotec, Oxford, UK). Both antibodies were diluted 1:100 in PBS–BSA–Sap.

The cells were washed with PBS–BSA–Sap for  $3 \times 5$  min and then incubated with a mixture of FITC conjugated F(ab')<sub>2</sub> fragment donkey anti-mouse IgG and a Dylight 594 conjugated F(ab')<sub>2</sub> fragment donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Europe Ltd, Suffolk, UK), both diluted 1:150. The cells were washed with PBS–BSA–Sap for  $3 \times 5$  min and finally rinsed with PBS. The cover slips were mounted on microscope slides with a fluorescent mounting medium (Dako) and viewed in a Nikon Eclipse 80i microscope. Pictures were taken with a Hamamatsu C5810 color intensified 3CCD camera.

### Calcium imaging

The astrocytes were incubated at room temperature with the Ca<sup>2+</sup>-sensitive fluorophore probe Fura-2/AM (Invitrogen Molecular Probes, Eugene, Oregon, USA) for 20 min (8  $\mu$ l in 990  $\mu$ l Hank's HEPES-buffered saline solution [HHBSS], containing 137 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.4 mM MgCl<sub>2</sub>, 1.26 mM CaCl<sub>2</sub>, 0.64 mM KH<sub>2</sub>PO<sub>4</sub>, 3.0 mM NaHCO<sub>3</sub>, 5.5 mM glucose, and 20 mM HEPES dissolved in distilled water, pH 7.4). The fluorophore probe was dissolved with 40  $\mu$ l of dimethyl sulfoxide (DMSO) and 10  $\mu$ l of pluronic acid (Molecular Probes, Leiden, The Netherlands). After incubation, the cells were rinsed three times with HHBSS before exposure to stimulators for 30 s. The cells were incubated with LPS (10 ng/ml) for 24 h. Naloxone (10<sup>−12</sup> M), endomorphin-1 (10<sup>−6</sup> M), and levetiracetam (10<sup>−4</sup> M) were applied 3.5 min before glutamate (10<sup>−4</sup> M). The experiments were performed at room temperature using a Ca<sup>2+</sup> imaging system with Simple PCI software (Compix Inc., Imaging Systems, Hamamatsu Photonics Management Corporation, Cranberry Twp, PA, USA) and an inverted epifluorescence microscope (Nikon ECLIPSE TE2000-E) with a 20 $\times$  (NA 0.45) fluorescence dry lens and a Polychrome V monochromator-based illumination system (TILL Photonics GMBH, Munich, Germany). The

various substances were applied using a peristaltic pump (Instech Laboratories, Plymouth Meeting, PA, USA) at an approximate rate of 600  $\mu$ l/min. One min after the start of the experiment, the stimulating substance was pumped into the pump tubes for 30 s. The substance took approximately 60 s to reach the cells through the tubes. HHBSS continued to flow through the pump tubes and onto the cells throughout the experiment. The images were captured with an ORCA-12AG (C4742-80-12AG) High-Res Digital Cooled CCD Camera (Hamamatsu Photonics Corporation).

The total areas under the curve (AUC), which reflects the amount of Ca<sup>2+</sup> released (Berridge, 2007), were analyzed to measure the vigor of the Ca<sup>2+</sup> response. The amplitude was expressed as the maximum increase of the 340/380 ratio. The area under the Ca<sup>2+</sup> peaks was calculated in Origin (Microcal Software Inc., Northampton, MA, USA). Forty cells were used for each experimental set up, taken from four different coverslips and from two different seeding times.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

The cells were rinsed twice in PBS and immediately lysed for 20 min on ice in cold radio-immunoprecipitation assay (RIPA) lysis buffer containing 150 mM NaCl, 1% IGEPAL<sup>®</sup> CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, (pH 8.0), supplemented with a protease inhibitor cocktail containing 104 mM AEBSF, 80  $\mu$ M aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A. The procedure was performed according to Persson et al. (2005). Separate aliquots were collected to determine the protein concentration. All of the samples were analyzed for the total protein content, and 20  $\mu$ g of the total protein from each sample was loaded into each lane of the gel.  $\beta$ -Actin was used as control for equal loading.

SDS–PAGE was conducted using the Novex pre-cast gel system (Invitrogen) according to the manufacturer's recommendations using 4–12% Bis-Tris gels (Invitrogen) at 200 V for 50 min. The separated proteins were transferred at 30 V for 60 min to a nitrocellulose membrane (Invitrogen) using NuPAGE transfer buffer (Invitrogen) supplemented with methanol and NuPAGE antioxidant. The membranes were rinsed twice with distilled water, and the proteins were visualized with Ponceau S solution (Sigma). The proteins were blocked with 5% fat-free skim milk (Semper AB, Sundbyberg, Sweden) in Tris-buffered saline (TBST; 50 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween) for 60 min at room temperature. The membranes were probed with a mouse monoclonal primary antibody against Na<sup>+</sup>/K<sup>+</sup>-ATPase ( $\alpha$ -subunit) diluted 1:250, washed 4 $\times$  for 2 min with TBST, followed with the secondary horseradish peroxidase (HRP)-conjugated antibody, donkey anti-mouse F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch) diluted 1:10,000, also washed several times in TBST. All of the primary and secondary antibodies were diluted in 5% fat-free skim milk in TBST. The antibody-bound protein was detected with an enhanced chemiluminescence kit (PerkinElmer Inc., Waltham, MA,



USA) and visualized using Fuji Film LAS-3000 (Tokyo, Japan).

### Actin visualization

The astrocyte cytoskeleton was stained with an Alexa488-conjugated phalloidin probe. Some of the cultures were incubated with 10 ng/ml LPS for 24 h. Other cultures were incubated with naloxone ( $10^{-12}$  M), endomorphin-1 ( $10^{-6}$  M), and/or levetiracetam ( $10^{-4}$  M) for 30 min before they were incubated with LPS and the proposed combination. The cultures were fixed with 4% paraformaldehyde and made permeable with PBS (Invitrogen) containing 1% BSA and 0.05% saponin followed by an Alexa568-conjugated phalloidin probe (Invitrogen) diluted 1:40 in PBS supplemented with 1% BSA. The coverslips were rinsed three times in PBS and mounted on microscope slides using Dako's fluorescent mounting medium (Dako) before being viewed with a fluorescence dry-objective lens attached to an inverted Nikon Optiphot-2 microscope.

### Cytokine release

The co-cultured astrocytes were stimulated for 24 h with 10 ng/ml LPS diluted in non-supplemented minimum essential medium (MEM) to measure IL-1 $\beta$  release. The inserts with endothelial cells were removed immediately before incubation. The cells were incubated with levetiracetam ( $10^{-12}$ – $10^{-4}$  M) or a combination of naloxone ( $10^{-12}$  M), endomorphin-1 ( $10^{-6}$  M), and levetiracetam ( $10^{-4}$  M) for 30 min prior to LPS treatment and remained in the medium throughout the experiment. The control (i.e., untreated) cells were maintained in non-supplemented MEM. The supernatants were collected for an enzyme-linked immunosorbent assay (ELISA), and the cells were analyzed for protein concentration.

Rat IL-1 $\beta$  (Nordic Biosite, Täby, Sweden) was used according to the manufacturer's instructions to measure the amount of cytokine with ELISA. Between every incubation step, several washes were performed. The protein concentration was determined as described below. The amount of IL-1 $\beta$  release was correlated to the amount of protein in each well.

### Protein determination

The protein determination assay was performed in accordance with the manufacturer's instructions using a detergent compatible (DC) Protein Assay (Bio-Rad, Hercules, CA, USA) based on the method used by Lowry et al. (1951) with minor modifications. The standard (0–4 mg/ml BSA) and samples were mixed with the reagents, incubated for 15 min at room temperature, read at 750 nm with a Versa-max microplate reader, and analyzed using SoftMax Pro 4.8 from Molecular Devices (Sunnyvale, CA, USA).

### Statistics

Differences across the different treatments were identified using a one-way analysis of variance (ANOVA) followed

by Dunnett's multiple comparisons test. The error bars represent the standard error of the mean (SEM).

## RESULTS

### Glutamate-evoked $\text{Ca}^{2+}$ responses were increased with LPS and attenuated by a combination of naloxone, endomorphin-1, and levetiracetam

The astrocytes were stimulated with glutamate ( $10^{-4}$  M), and  $\text{Ca}^{2+}$  imaging experiments were performed on 15- to 17-day-old co-cultured astrocytes. The endothelial cell inserts were removed just before the experiments. All of the cells responded,  $n = 40$ . The AUCs were calculated, and the number of peaks was counted (Fig. 2A, B).

The astrocytes incubated with LPS (10 ng/ml) for 24 h showed an increase in glutamate-evoked  $\text{Ca}^{2+}$  signaling. The AUC was significantly increased ( $P < 0.001$ ) as well as the number of peaks. All cells responded,  $n = 40$  (Fig. 2A, B). Endomorphin-1 attenuated the glutamate signaling ( $P < 0.05$ ), and all cells responded,  $n = 40$  (Fig. 2A, B). Naloxone ( $10^{-12}$  M) alone or together with endomorphin-1 ( $10^{-6}$  M) did not attenuate the glutamate-upregulated  $\text{Ca}^{2+}$  oscillations,  $n = 40$  (Fig. 2A, B). Levetiracetam ( $10^{-4}$  M) attenuated the glutamate signaling ( $P < 0.001$ ), and all cells responded,  $n = 40$  (Fig. 2A, B). The combination of naloxone, endomorphin-1, and levetiracetam attenuated the glutamate signaling ( $P < 0.001$ ), and all cells responded (Fig. 2A, B).

The results show that a combination of endomorphin-1, naloxone in ultralow concentration, and levetiracetam fully attenuated the LPS-induced glutamate  $\text{Ca}^{2+}$  transients.

### $\text{Na}^+/\text{K}^+$ -ATPase expression was decreased by LPS and increased by a combination of naloxone, endomorphin-1 and levetiracetam

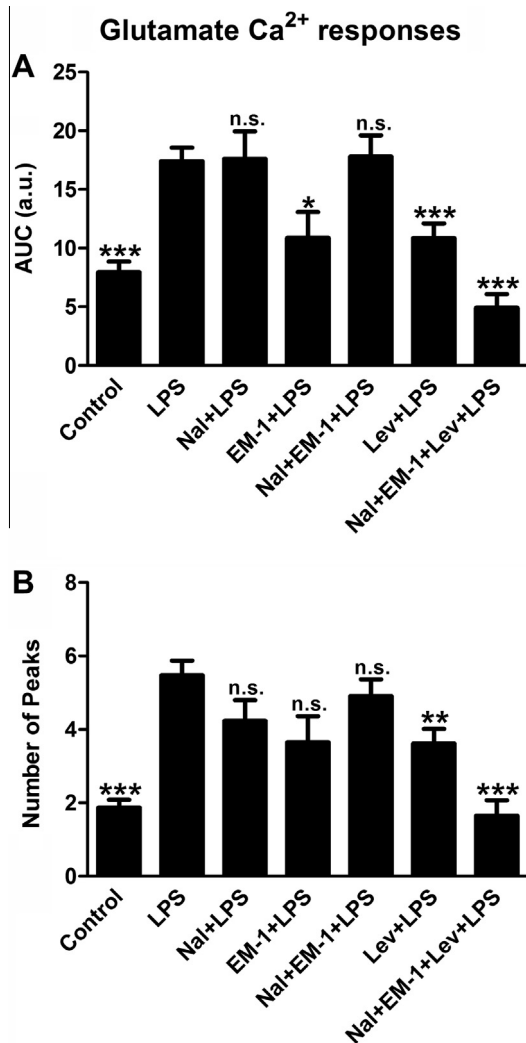
The expression of  $\text{Na}^+/\text{K}^+$ -ATPase for cultures incubated with LPS for 24 h was studied using Western blot analysis. The expression of  $\text{Na}^+/\text{K}^+$ -ATPase was decreased ( $P < 0.05$ ) in the cultured cells and was increased when the cells were incubated with a combination of naloxone, endomorphin-1, and levetiracetam ( $P < 0.01$ ,  $n = 6$ , Fig. 3A). None of these agents alone showed any effect on the expression of  $\text{Na}^+/\text{K}^+$ -ATPase (Fig. 3B).

These results show that a combination of endomorphin-1, naloxone in ultralow concentration, and levetiracetam increased the  $\text{Na}^+/\text{K}^+$ -ATPase activity.

### IL-1 $\beta$ release was decreased by levetiracetam

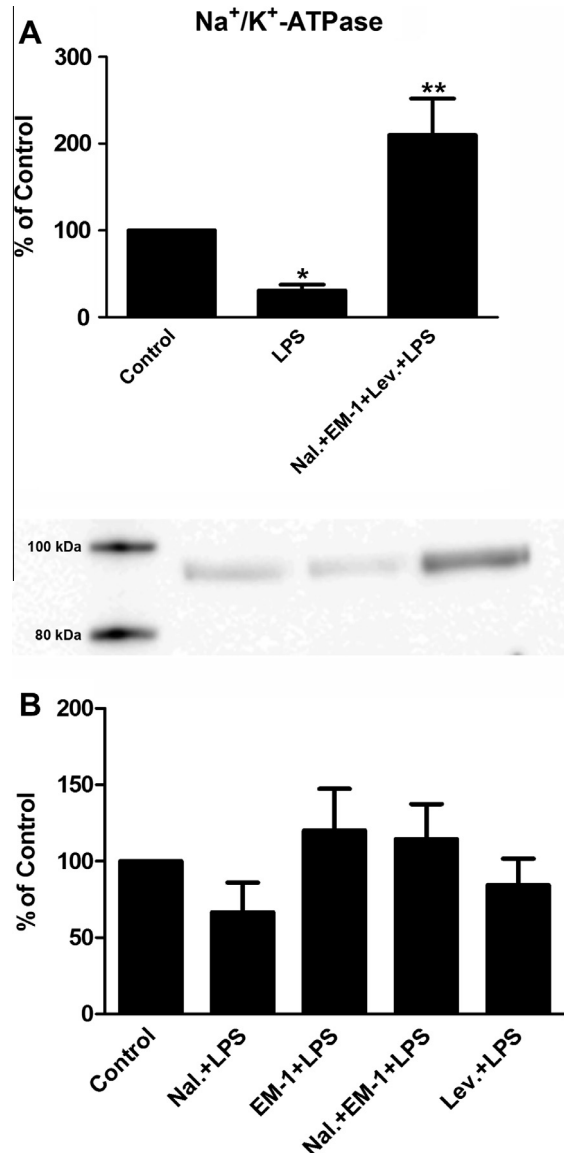
The cultures were pre-incubated with levetiracetam ( $10^{-12}$ – $10^{-4}$  M) 30 min prior to incubation with LPS (10 ng/ml) and levetiracetam for 24 h. Levetiracetam ( $10^{-4}$  M) reduced the LPS-induced increase in IL-1 $\beta$  ( $P < 0.05$ ,  $n = 4$ , Fig. 4). IL-1 $\beta$  release after LPS stimulation was  $4.3 \pm 0.5$  ng/ml and was set to 100%.

The cultures were pre-incubated with a combination of naloxone ( $10^{-12}$  M), endomorphin-1 ( $10^{-6}$  M), and



**Fig. 2.** Glutamate-evoked  $\text{Ca}^{2+}$  oscillations. All cells were stimulated in a  $\text{Ca}^{2+}$  imaging system. The cells were incubated with a  $\text{Ca}^{2+}$ -sensitive fluorophore probe (Fura-2/AM). Glutamate ( $10^{-4}$  M) elicited  $\text{Ca}^{2+}$  responses in the astrocytes and was used as control. (A) The areas under the  $\text{Ca}^{2+}$  peaks (AUC) of the  $\text{Ca}^{2+}$  transients were calculated. The cells incubated with LPS (10 ng/ml) for 24 h showed increased  $\text{Ca}^{2+}$  signaling in the form of oscillations. The cells pre-incubated with a combination of naloxone (Nal) ( $10^{-12}$  M), endomorphin-1 (EM-1) ( $10^{-6}$  M), and levetiracetam (Lev) ( $10^{-4}$  M) for 30 min before they were incubated with a combination of LPS (10 ng/ml), Nal, EM-1, and Lev for 24 h showed a significant decrease in the glutamate-evoked  $\text{Ca}^{2+}$  transients. Cells pre-incubated with Nal, a combination with Nal and EM-1, for 30 min before they were incubated with a combination of LPS and the respective substances for 24 h did not attenuate the glutamate-upregulated  $\text{Ca}^{2+}$  transients. Cells pre-incubated with EM-1 or Lev attenuated the glutamate-upregulated  $\text{Ca}^{2+}$  transients. (B) The number of peaks was counted for each cell. Statistical analysis: The level of significance was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test. \* $P < 0.05$ , \*\*\* $P < 0.001$ , n.s. = not significant.

levetiracetam ( $10^{-4}$  M) 30 min prior to incubation with LPS (10 ng/ml), naloxone, endomorphin-1, and levetiracetam for 24 h. The LPS-induced increase in IL-1 $\beta$  was decreased ( $P < 0.001$ ,  $n = 4$ , Fig. 5). Naloxone alone did not decrease the IL-1 $\beta$  release (Forshammar et al., 2011) nor did endomorphin-1 or a combination of naloxone and endomorphin-1. Data are not shown.

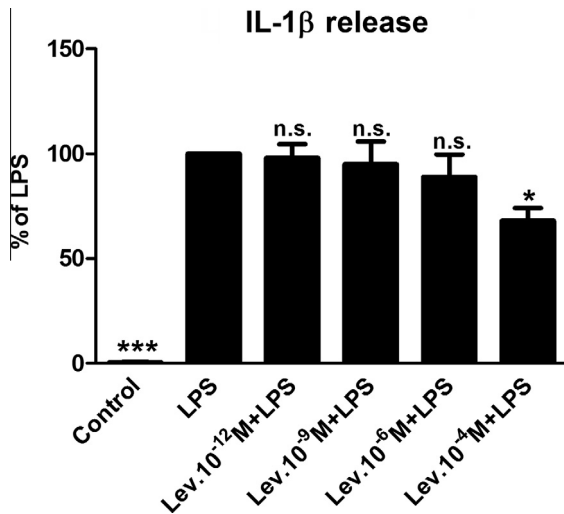


**Fig. 3.** Na<sup>+</sup>/K<sup>+</sup>-ATPase expression. The cultures were incubated with LPS (10 ng/ml) for 24 h. The Na<sup>+</sup>/K<sup>+</sup>-ATPase expression was decreased compared with controls. (A) The cells were pre-incubated with a combination of naloxone (Nal) ( $10^{-12}$  M), endomorphin-1 (EM-1) ( $10^{-6}$  M), and levetiracetam (Lev) ( $10^{-4}$  M) for 30 min before they were incubated with a combination of LPS (10 ng/ml), Nal, EM-1, and Lev for 24 h. The Na<sup>+</sup>/K<sup>+</sup>-ATPase expression increased with the cocktail. Na<sup>+</sup>/K<sup>+</sup>-ATPase appears as a band just below 110 kDa. (B) Nal, EM-1 or Lev alone did not show any effect. Statistical analysis: The level of significance was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test. \*\* $P < 0.01$ , \* $P < 0.05$ .  $n = 6$ .

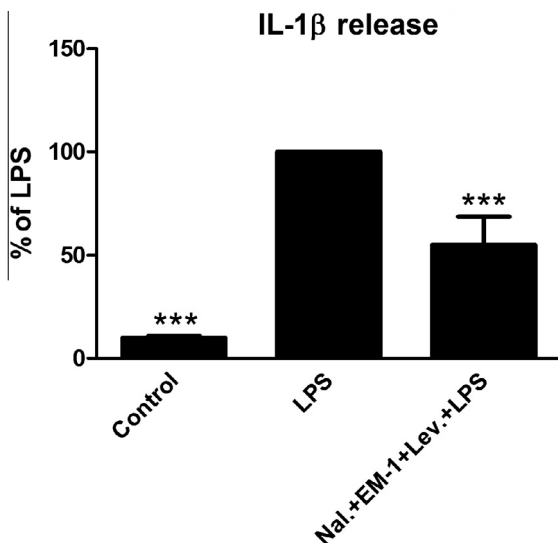
The results show that levetiracetam decreased the IL-1 $\beta$  release, and that the combination of the three drugs did not have any further influence on the release.

### Actin filaments

The astrocytes were stained with an Alexa488-conjugated phalloidin probe. The untreated cultures were dominated by F-actin organized in stress fibers (control). The cultures incubated with LPS (10 ng/ml) for 24 h showed



**Fig. 4.** IL-1 $\beta$  release. The cultures were pre-incubated with levetiracetam (Lev) ( $10^{-12}$ – $10^{-4}$  M) 30 min prior to incubation with LPS (10 ng/ml) and Lev for 24 h. Lev ( $10^{-4}$  M) reduced the LPS-increased IL-1 $\beta$  concentration. Statistical analysis: The level of significance was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test. \* $P < 0.05$ .  $n = 6$ .



**Fig. 5.** IL-1 $\beta$  release. The cultures were pre-incubated with a combination of naloxone (Nal) ( $10^{-12}$  M), endomorphin-1 (EM-1) ( $10^{-6}$  M), and levetiracetam (Lev) ( $10^{-4}$  M) 30 min prior to incubation with LPS (10 ng/ml) and Nal + EM-1 + Lev for 24 h. The LPS-induced increase in IL-1 $\beta$  was decreased. Statistical analysis: The level of significance was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test. \*\*\* $P < 0.001$ .  $n = 6$ .

a more diffuse organization of the actin filaments, and the ring structures were more pronounced. Naloxone ( $10^{-12}$  M) as well as a combination of naloxone and endomorphin-1 ( $10^{-6}$  M) prevented the restructuring of the actin filaments (Fig. 6). The cells were pre-incubated with a combination of naloxone, endomorphin-1, and levetiracetam ( $10^{-4}$  M) for 30 min before they were incubated with a combination of LPS (10 ng/ml), naloxone, endomorphin-1, and levetiracetam for 24 h. This combination prevented the restructuring of the actin

filaments even more robustly (Fig. 6). Levetiracetam can also prevent the restructuring of actin filaments in the cytoskeleton; however, the combination of the three substances showed the most optimal result.

The results show that the combinations of naloxone and endomorphin-1, as well as naloxone, endomorphin-1 and levetiracetam equally prevented the restructuring of actin filaments.

## DISCUSSION

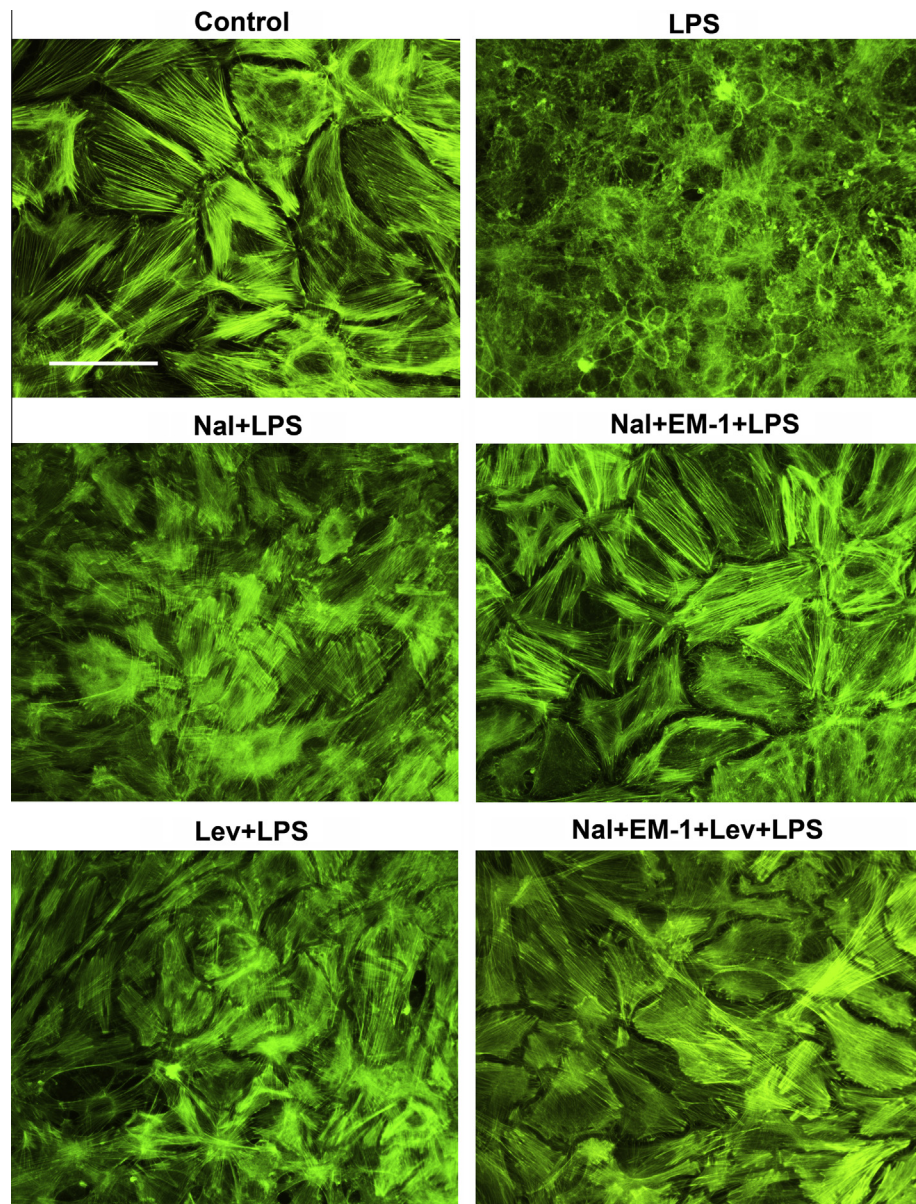
We have identified four parameters,  $\text{Ca}^{2+}$  signaling in the astrocyte networks,  $\text{Na}^+/\text{K}^+$ -ATPase activity, IL-1 $\beta$  release, and actin filament organization, which take part in mechanisms underlying or contributing to neuroinflammation. The astrocytes are potential targets during the inflammatory state (Forshammar et al., 2011; Block et al., 2012). To achieve the full protective effects, a combination of treatments is required. It seems important to maintain the extracellular concentration of pro-inflammatory cytokines, such as IL-1 $\beta$ , at a low level, which can be accomplished with levetiracetam. In addition, it seems important to reduce the increased extracellular glutamate concentration, which stimulates an increased propagation of astrocyte intercellular  $\text{Ca}^{2+}$  waves. This is partly possible with endomorphin-1 and ultralow concentrations of naloxone. The low protein level and activity of  $\text{Na}^+/\text{K}^+$ -ATPase has to be increased, which can be achieved through a decreased activation of the  $\text{G}_s$  protein and an increased activation of the  $\text{G}_{i/o}$  protein. The actin filaments, which are disorganized, will be restored. However, to achieve these features, a combination of these substances is necessary for the restoration of the cellular parameters disrupted by inflammation.

During inflammation and when cells are induced with inflammatory agents,  $\text{Ca}^{2+}$  signaling in the astrocyte networks is disturbed. There is an increased release of IL-1 $\beta$ , which results in gap junction inhibition (Kim et al., 2010). There is also an increased release of ATP, which provides a parallel system for intercellular  $\text{Ca}^{2+}$  communication between astrocytes (Fields and Burnstock, 2006) and triggers the release of glutamate (Domercq et al., 2006).

The inflammatory inducer LPS changed the glutamate-evoked  $\text{Ca}^{2+}$  transients from single peaks to  $\text{Ca}^{2+}$  oscillations when the astrocytes were incubated with LPS for a long time. This means that the intracellular  $\text{Ca}^{2+}$  release from the endoplasmic reticulum increased, which leads to an overstimulation of the  $\text{G}_q$  mGluR5-dependent astrocyte  $\text{Ca}^{2+}$  elevations followed by the propagation of intercellular  $\text{Ca}^{2+}$  waves (Bradley et al., 2009). Another cellular parameter that is altered by LPS is  $\text{Na}^+/\text{K}^+$ -ATPase, which results in the downregulation and disorganization of actin filaments (Forshammar et al., 2011).

The challenge is to reverse inflammation-induced changes to return cellular homeostasis back to normal physiological levels (Watkins and Maier, 2003; Hansson, 2006, 2010; Zhuo et al., 2011). Several substances have been suggested for this purpose, and a huge





**Fig. 6.** Actin filaments. The astrocytes were stained with an Alexa488-conjugated phalloidin probe. The untreated culture was dominated by F-actin organized into stress fibers (control). The cultures incubated with LPS (10 ng/ml) for 24 h showed a more diffuse organization of actin filaments, and the ring structures were more pronounced. Naloxone ( $10^{-12}$  M) as well as a combination of naloxone and endomorphin-1 (EM-1) ( $10^{-6}$  M), or levetiracetam (Lev) ( $10^{-4}$  M) prevented the restructuring of actin filaments. Cells pre-incubated with a combination of Nal, EM-1, and Lev for 30 min before they were incubated with a combination of LPS (10 ng/ml), Nal, EM-1, and Lev for 24 h prevented also the restructuring. Scale bar = 50  $\mu$ m. Representative images are presented from at least three different seedings.

number of experiments have been performed to find an agent that possesses all the properties necessary to restore inflammation-reactive cells (Tchivileva et al., 2009; Nadeau et al., 2011; Wen et al., 2011).

The CNS is sensitive to opiates, and endogenous opioids modulate cellular proliferation. The classical opioid system is best known for its effects on nociception (Fields, 2004); however, it is also important for modulating the immune system and releasing  $\text{Ca}^{2+}$  from intracellular stores. These effects are mediated by G protein-coupled opioid receptors (Uhl et al., 1994; Hansson et al., 2008), which can be activated by inflammatory stimuli (Chen and Marvizón, 2009;

Vanderah, 2010). It is known that  $\mu$ -opioid receptors preferentially couple to pertussis toxin (PTX)-sensitive G proteins,  $\text{G}_i/\text{G}_o$ , and inhibit the adenylyl cyclase/cAMP pathway (Connor and Christie, 1999). A switch in G protein coupling from  $\text{G}_i/\text{G}_o$  to  $\text{G}_s$  proteins, which activates the adenylyl cyclase/cAMP pathway, has been shown to be elicited by the chronic administration of opioids in dorsal root ganglion neurons in culture (Crain and Shen, 1995) and *in vivo* in rats (Wang et al., 2005). The attenuation of  $\text{G}_i/\text{G}_o$  protein coupling is involved in the initiation and/or maintenance of allodynic pain (Womer et al., 1997). Therefore, it seems important to stimulate the  $\text{G}_i/\text{G}_o$  proteins and decrease the  $\text{G}_s$

protein activities. Naloxone in ultralow concentrations inhibited the  $\mu$ -opioid receptor/ $G_s$  coupling and stimulated the  $\mu$ -opioid receptor  $G_i/G_o$  protein (Tsai et al., 2009). Ultralow doses of naloxone in combination with morphine attenuate chronic morphine-induced  $G_s$  protein coupling in rats (Shen and Crain, 1997; Wang et al., 2005), whereas higher concentrations antagonized excitatory and inhibitory opioid receptors (Crain and Shen, 1995). Ultralow concentrations of naloxone also inhibit the disturbed astrocyte activation (Forshammar et al., 2011; Block et al., 2012). These results indicate that ultralow doses of naloxone in combination with a  $\mu$ -opioid receptor agonist, such as morphine or endomorphin-1, increase  $G_i/G_o$  protein coupling and suppress neuroinflammation by reducing the excitatory  $G_s$  protein-coupled opioid receptors in rats (Tsai et al., 2009), patients (Maxwell et al., 2005), and astrocyte cultures (Block et al., 2012). This combination switches the  $\mu$ -opioid receptor coupling from  $G_s$  protein to  $G_i/G_o$  protein, and ultralow concentration of naloxone prevents the switch back again.

Downregulation of  $Na^+$  transporters has been observed in different inflammatory cellular systems, which indicates a dysfunction of  $Na^+/K^+$ -ATPase activity and can lead to increased intracellular  $Na^+$  concentrations (Schmidt et al., 2007). Astrocytes have a strong resistance to  $Na^+$  influx only when  $Na^+/K^+$ -ATPase activity is maintained (Takahashi et al., 2000).

$Na^+/K^+$ -ATPase is stimulated by low concentrations of naloxone by the activation of the Scr/epidermal growth factor receptor (EGFR) intracellular pathway, which increases the intracellular  $Ca^{2+}$  concentration through the phospholipase C/inositol 1,4,5-trisphosphate receptor ( $IP_3$ ) pathway (Zhang et al., 2008). Ultralow concentrations of naloxone prevented the inflammation-induced downregulation of  $Na^+/K^+$ -ATPase in astrocytes, and stimulated the intracellular  $Ca^{2+}$  release (Forshammar et al., 2011). Data indicate that endomorphin-1 as well as morphine indirectly stimulates  $Na^+/K^+$ -ATPase activity by activating  $\mu$ -opioid receptors and  $G_i/G_o$  proteins (Masocha et al., 2002; Horvath et al., 2003). This suggests that the combination of ultralow concentrations of naloxone and endomorphin-1 or morphine, stimulates  $G_i/G_o$  protein and  $Na^+/K^+$ -ATPase activities.

The involvement of the cytoskeleton in controlling the plasma membrane microdomains and endoplasmic reticulum complex seems to be of importance. The adaptor protein ankyrin B is associated with the  $Na^+$  pump and with endoplasmic reticulum proteins, such as the  $IP_3$  receptor. The main cytoplasmic matrix of proteins, spectrin and actin, are attached to ankyrin B. An intact cytoskeleton is required for the propagation of astrocyte  $Ca^{2+}$  waves (Cotrina et al., 1998; Forshammar et al., 2011), and disruption abolishes the  $Ca^{2+}$  oscillations by changing the balance between the  $Ca^{2+}$  regulating processes (Sergeeva et al., 2000), which is observed when astrocytes are affected by LPS.

Naloxone at ultralow concentrations prevented the restructuring of the actin filaments in the cytoskeleton of astrocytes (Forshammar et al., 2011). Filamin A,

cross-linking cytoplasmic actin filaments, has been found to regulate cell signaling by interacting with several receptors including  $\mu$ -opioid receptors. Naloxone binds to a pentapeptide segment of filamin A, and in ultralow concentrations naloxone prevents morphine's coupling to the  $G_s$  protein (Wang and Burns, 2009). This shows the importance of a working  $Na^+$  pump, which is coupled to the cellular cytoskeleton.

The biosynthesis of IL-1 $\beta$  is complex and regulated at multiple levels. The increased release of IL-1 $\beta$  observed under inflammatory conditions reduces intercellular communication by the passage of  $IP_3$  through gap junctions in astrocyte networks (Retamal et al., 2007). Therefore, neuroprotective  $Ca^{2+}$  signaling is inhibited. As a result, the other  $Ca^{2+}$  signaling pathway, the extracellular ATP pathway, increases (Froger et al., 2010).

A substance that has been observed to decrease IL-1 $\beta$  release is the anti-epileptic drug levetiracetam (Keppra). Levetiracetam has anti-inflammatory properties and has been shown to restore functional gap junction coupling in astrocytes under inflammatory conditions (Haghikia et al., 2008). Furthermore, levetiracetam had the ability to decrease the enhanced IL-1 $\beta$  level (Haghikia et al., 2008). Levetiracetam can also normalize the membrane resting potential, and prevent the restructuring of actin filaments in the cytoskeleton (Stienen et al., 2010). We found also a decrease of the enhanced IL-1 $\beta$  level at higher concentrations, and restoration of the actin filaments. Microglia were in fact still minor after treatment with LPS for 24 h, evidenced by a sparse OX42 staining, and the production of IL-1 $\beta$  can be in majority attributed to astrocytes.

Levetiracetam restored the increased  $Ca^{2+}$  oscillations; however, in combination with endomorphin-1 and an ultralow concentration of naloxone, the effect was more pronounced. Similar results were observed regarding  $Na^+/K^+$ -ATPase. Concerning the IL-1 $\beta$  release, naloxone alone (Forshammar et al., 2011) or in combination with endomorphin-1 did not decrease cytokine release. However, levetiracetam had some restoring effects on the actin filaments. In the absence of a working astrocyte network, interactions between neurons, astrocytes, and other cell types will be problematic. The combination of ultralow concentrations of naloxone, endomorphin-1, and levetiracetam, switches the activation of the  $\mu$ -opioid receptor to  $G_{i/o}$  protein coupling, which stimulates the  $Na^+/K^+$ -ATPase activity and restores the actin filaments and the intercellular  $Ca^{2+}$  signaling. The extracellular  $Ca^{2+}$  signaling decreases and so does the inflammatory enhanced IL-1 $\beta$  release. These actions will lead to neuroprotective intercellular  $Ca^{2+}$  signaling in the astrocyte networks and will restore cellular homeostasis.

## CONCLUDING REMARKS

The present results are based on the findings that the combination of a  $\mu$ -opioid receptor antagonist at ultralow concentrations, a  $\mu$ -opioid receptor agonist, and an



agent attenuating IL-1 $\beta$  release can restore the cellular parameters altered by inflammation to the normal, non-inflammatory state. This three-drug composition could be clinically useful for treatment of long-term pain.

## CONFLICT OF INTEREST

There is no conflict of interest.

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